Master's Thesis

Identifying Inter-Domain Dynamics of Pin1 With Single Molecule FRET

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2018

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A thesis
submitted to the Graduate School of UNIST
in partial fulfillment of the
requirements for the degree of
Master of Science

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12 / 8 / 2017
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ABSTRACT

Pin1, Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1, contains two functional domains, WW domain for the recognition of the peptide substrate and PPIase domain for *cis-trans* isomerization, linked by a flexible, intrinsically disordered region (IDR). Recent evidences from NMR studies suggest the inter-domain migration in this bivalent protein, the mechanism of which remains puzzling. Therefore, we characterized the inter-domain dynamics of Pin1 from multi-color single molecule fluorescence resonance energy transfer measurements. We measured the dynamics of Pin1 directly tethered to the surface or co-encapsulated in a lipid vesicle with the peptide substrate in order to observe its transient interaction with the substrate. We visualized the structural dynamics between the Pin1 domains as well as their interaction with the peptide substrate in real time to reveal the temporal correlation between the domain recognition and isomerization dynamics. This approach will provide new understanding of the role of the intrinsically disordered regions in coordinating multi-domain protein functions.

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NOMENCLATURE

Pin1 : Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

PPIase : peptidyl-prolyl isomerase

FRET : Fluorescence Resonance Energy Transfer

Cy3, Cy5 : Cyanine 3, Cyanine 5

TIRF : Total Internal Reflect Fluorescent

IDR : Intrinsically Disordered Region

PEG : Poly Ethylene Glycol

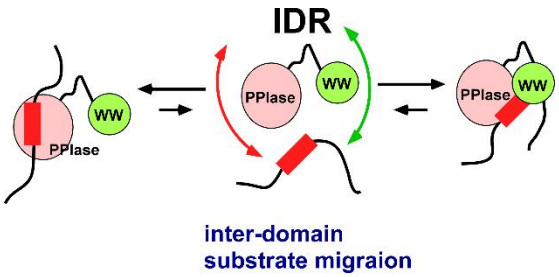
NMR : nuclear magnetic resonance

Contents

I. Introduction

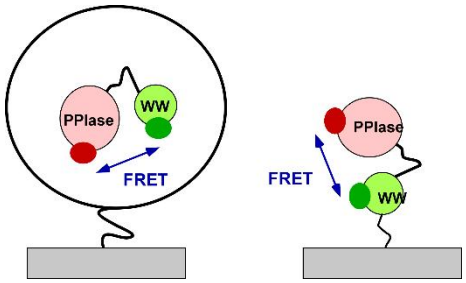
Intrinsically Disordered Proteins (IDP), natively unfolded proteins in other words, have some non-identified regions and frequently found in eukaryotic cells, studied to be related to cellular regulation, signaling including transcription, translation and cell cycles. This 'disordered' conformation is formed commonly due to small level of sequence complexity or low contents of bulky hydrophobic amino acids. This leads into the interesting figure of IDP; they are unable to fold or to form into stable conformation spontaneously but dynamically disordered and fluctuate rapidly. As a result, IDP takes critical roles in some human diseases such as Alzheimer's disease or cancer. However, while the architectures are frequently found, the roles of IDP or linkers are still veiled. This experiment intents to characterize the inter-domain migration mechanism of Pin1, peptidyl-prolyl cis/trans isomerase (PPIase), on its peptide substrates on single molecule level, with understanding real time dynamics.

This project is to study the role of intrinsically disordered regions(IDRs) connecting the two functional domains in Pin1; N-terminal (WW domain) specifically binds to the phosphor-peptide having phosphorylated Ser/Thr-Pro dipeptide motif, while C-terminal has enzyme activity to isomerize Pro peptide bond in the above dipeptide motif. Both domains bind to pSer/pThr-Pro motif with different affinity that WW domain binds with stronger affinity over PPIase. In a native consideration, existing the higher affinity domain tethered to the enzyme domain inhibits the substrate access to the PPIase domain and therefore it reduces the isomerization activity of Pin1. This rose us to think over the functional significance of the architecture having two domains with intervening IDR between them. Pin1 showed the 1,000 enhanced affinity to the bivalent peptide over the monovalent substrates each of which has sole binding sites in the bivalent peptide from NMR studies. This supports that the affinity enhancement is not ascribed to the simultaneous binding of the domains to the sites in the bivalent peptide, instead the peptide migration between the domains has increased the affinity. The substrate released from the domain is readily captured by the other domain is expected in the inter-domain substrate migration, so the substrate stays in bound to Pin1 with longer residence time than the cases in binding to each isolated domain.



[Fig. 1] inter-domain substrate migration
Left scheme shows alternative dynamics of domains of pin1 on substrate while right scheme explains originally known dynamics of pin1.

To observe this inter-domain substrate migration, smFRET, single molecule Fluorescence Resonance Energy Transfer, is counted as one of the most powerful tool to understand very direct and



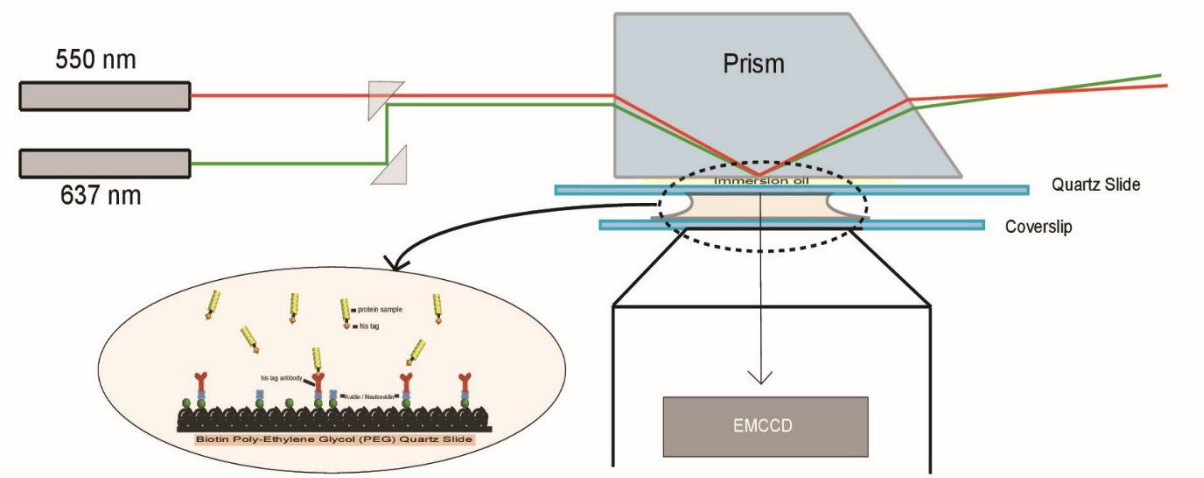
[Fig. 2] vesicle FRET experiment scheme
Tethering sample on slide(right) might distort the native dynamics of protein domains, but vesicle design (left) can show naïve dynamics of protein.

eidetic understanding on molecule dynamics. With ordinary optics, there were high limitation on optics to observe biological / chemical phenomena; low resolution level, high background which distort or bother real signal of interest. When using sm FRET on TIRF microscope, Total Internal Reflection Fluorescence microscope, these problems can be solved; possible to understand dynamics until few nanometer's dynamics and background free imaging by applying evanescent field. This method is frequently used in many fields to figure out, to list some, dynamics of RNA folding and catalysis, non-canonical DNA dynamics, ion channels or signal transductions. Therefore, in this project, using this smFRET to identify inter-domain migration. There is one concern still that disturbance in the intrinsic domain dynamics by tethering the protein to the plate. To solve it, we use vesicle system on FRET to see more natural dynamics of this dynamics. [(1), - (3)]

II. Methods and Materials

2.1. TIRF and FRET

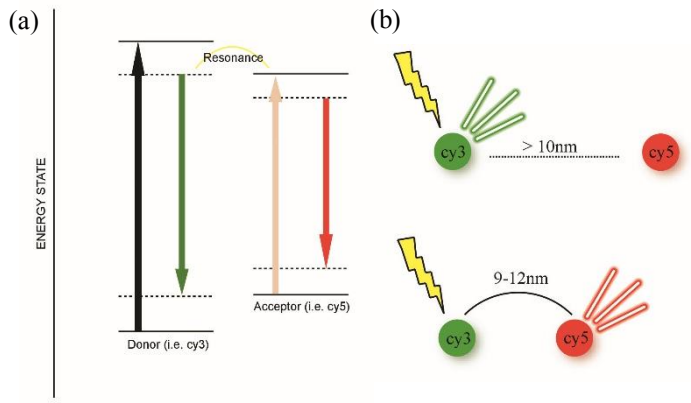
Many cellular and molecular biological phenomena with dynamics have been studied with conventional fluorescence microscopes. However, fluorophores in surrounding medium, which are off the target to observe, also stays in equilibrium state. This may cause unwanted critical problem on imaging due to excited molecules from surroundings, leading into high intensity of background which bothers to analyze the molecules of interests only. Here, we used TIRF, Total Internal Reflection Fluorescence, microscope which can solve this problem by enabling selective excitation of sample, only surface-bound molecules; this molecule exiting area is called 'evanescent field'. The field is



[Fig. 3] TIRF microscopy

The scheme shows simplified pathway of lasers to excite dye labeled in target substance, protein or DNA. It enters into prism, penetrate till quartz slide which has same reflection index, and evanescent field is formed where total internal reflection occurs. It excites targeted molecules on quartz slide, other labelled proteins on surroundings which are off-the-target, will not be able to be excited, hence giving no background signals. Surface of quartz is PEGylized with Biotin-PEG(Poly-Ethylene Glycol), attaches with sample with antibody reaction whose antibody is attached on PEGylized quartz with neutravidin-biotin interaction.

created only with total internally reflection, as in prism to sample pathway of light. The field decays exponentially from the interface, where quartz slide and sample meets, and thus it penetrates only up to 100nm of sample. This makes only surface-attached sample excited, therefore, high efficiency to reduce problems which can be caused by background, so that it can be considered one of the powerful tool to study single molecule level of researches.



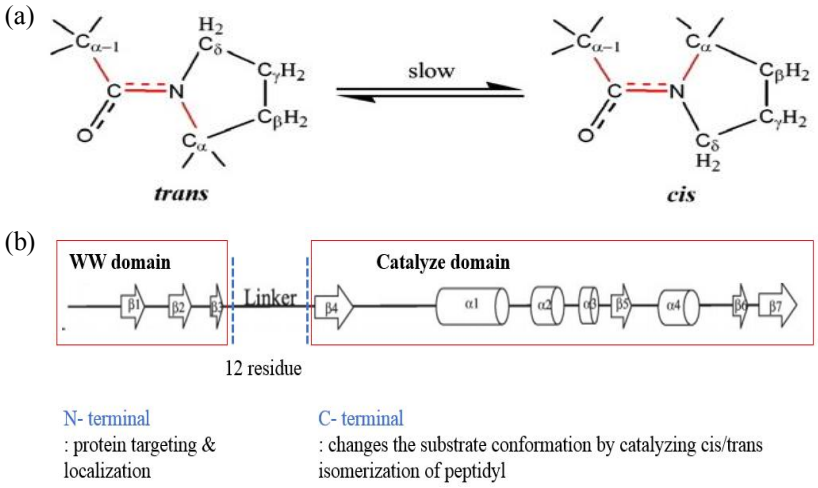
[Fig. 4] FRET

(a) FRET occurs with energy transfer from donor's excitation as acceptor's emission wavelength, eventually shows acceptor's emission wavelength. (b) Within approximately 10nm, FRET occurs which demonstrates spatial information of two(or more) target molecules.

FRET, Fluorescence Resonance Energy Transfer, has known to well-studied method to study single molecule dynamics, and as well to structural studies to the experimental arsenal of protein dynamic investigators. In FRET measurement, non-radiative energy transfer occurs between donor and acceptor dye molecules, when there is intervening distance which can be measured from the ratio of acceptor to total emission intensity. Therefore, conformational dynamics of single molecules are able to measure in real-time level. It is indeed useful and trustful method in revealing population distribution of inter-dye distances.[4]

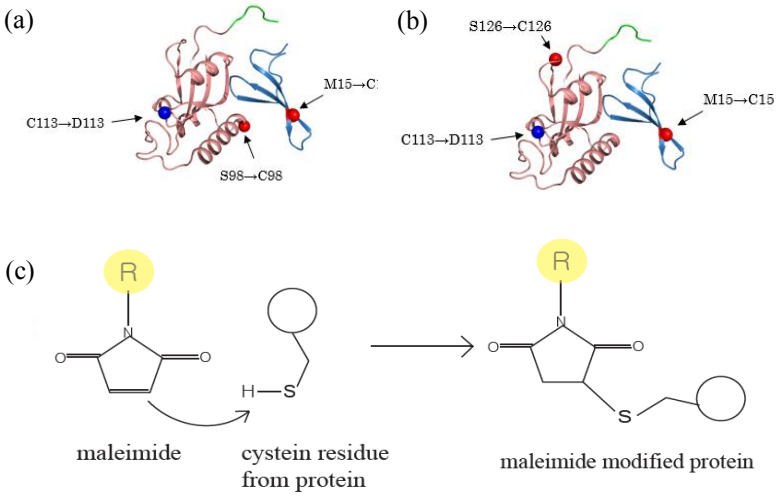
2.2. Sample preparation and purification

Therefore, labeling donor/acceptor dye into appropriate position on protein is essential for this experiment. So, to understand approximate conformation about the protein of interest is also highly asked on this stage. Therefore, I would like to introduce about protein what is studied during this experiment, firstly, then labeling methods on the protein. PPIase, peptidyl prolyl isomerase, is *cis/trans* isomerase on proline. All amino acids stay in *trans*/*cis* state – mostly staying in *trans* conformation due to steric hindrance, making *trans* state more stable, usually *trans* state is more than 100 times strongly favored than *cis* form. However, proline and its preceding amino acid are exceptional, which has peptidyl-prolyl bond. In this case, *trans* isomer is only 'slightly' favored, resulting in *trans* and *cis* conformation existing together in media, where *cis* conformation takes about



[Fig. 5] Pin1, as PPIase, has unique conformation
(a) PPIase has relatively higher ratio of trans form due to proline residue, unlike other amino acid residues. (b) Pin1 has WW domain which targets the enzyme activity site and Catalyze domain which actually isomerize *cis/trans* formation

up to 30%. And among these PPIases, Pin1 are the only known phosphorylation-dependent PPIase that isomerize on the p(Ser/Thr)-Pro motifs, which targets for man protein kinases. And this Pin1 is known to have 2 domains, WW domain on N terminal, which indicates the substrate, and catalytic domain on C terminal, which actually do enzymatic activity, and these two domains are connected with loop, in other words, linker. In this experiment, we want to see inter-domain dynamics from these two domains through linker, so we modified specific sites with cysteine so that maleimide dyes can be labelled. The protein is prepared by University of Hiroshima, with lyophilized state, with 98C modified and 126C modified sample. Each protein were incubated on RT for 15-20 mins, with ratio of n(protein) : n(cy3) : n(cy5) = 1 : 5 : 10, while n(A) stands for mole of substrate A, not concentration. This ratio has set by few understandings of labeling dyes on protein for FRET purpose; (1) the number of accepter dye should be more than the number of donor, (2) the number of dyes should be concentrated enough to ensure all labeling residues (i.e. cysteine) of protein can react with target dyes (i.e. maleimide). After incubation on RT, the labeled proteins are expected to be filtered by using P6

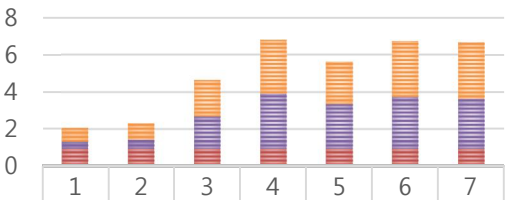


[Fig. 6] labeling maleimide on specific position on Pin1

98 position and 126 position amino acid Serine is modified into cysteine each to attach maleimide dyes, while 113 positioned cysteines are modified into aspartic acids to prevent unnecessary labelings (a),(b). (c) It shows chemical reaction how maleimide and cthiol group from cysteine residues form chemical bonds.

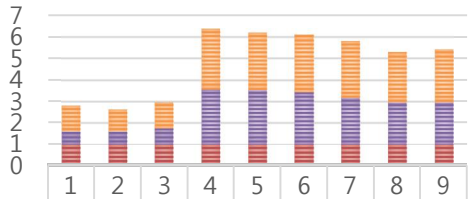
column, which filter samples based on weight, whose exclusion limit of gel is from 1,000 to 6,000 Da. Since Pin1's MW is 20,406 Da, while cy3 and cy5 maleimide's is 765.95 Da and 791.95 Da, it is expected that there should be significant step when labeled protein is filtered and free dyes starts to leak with labeled dye. From the following chart, it is shown that first 2 columns (for 98C spin column (2), until third column is considered) have [protein] : [cy3] : [cy5] = 1 : 1> : 1>, where [A] stands for

(a) 98C PIN1 SPIN COLUMN (1)



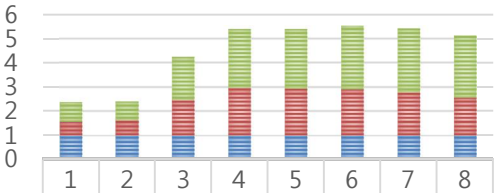
	1	2	3	4	5	6	7
cy5	0.716	0.849	1.932	2.881	2.269	2.975	3.012
cy3	0.339	0.456	1.684	2.904	2.333	2.713	2.62
pin1	1	1	1	1	1	1	1

(b) 98C PIN1 SPIN COLUMN (2)



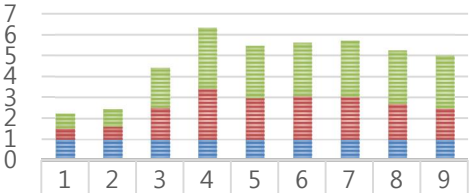
	1	2	3	4	5	6	7	8	9
cy5	1.2	1	1.18	2.81	2.64	2.67	2.64	2.34	2.48
cy3	0.61	0.62	0.77	2.55	2.54	2.42	2.17	1.96	1.94
pin1	1	1	1	1	1	1	1	1	1

(c) 126C PIN1 SPIN COLUMN (1)



	1	2	3	4	5	6	7	8
cy5	0.81	0.79	1.79	2.41	2.44	2.6	2.65	2.55
cy3	0.56	0.61	1.44	1.97	1.93	1.91	1.76	1.55
pin1	1	1	1	1	1	1	1	1

(d) 126C PIN1 SPIN COLUMN (2)



	1	2	3	4	5	6	7	8	9
cy5	0.73	0.82	1.89	2.92	2.49	2.56	2.68	2.53	2.5
cy3	0.51	0.63	1.52	2.4	1.97	2.05	2.03	1.7	1.48
pin1	1	1	1	1	1	1	1	1	1

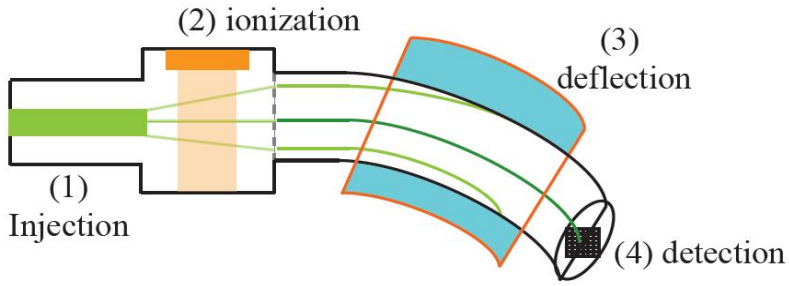
[Chart.1] chart for filtered 98C, 126C pin1 protein after labeling incubation

This chart shows relative concentration of dye, cy3 and cy5, upon concentration of pin1. Y-axis shows accumulated value of protein, cy3 and cy5; X-axis means turn of washed off solutions from incubation of mix with dyes and protein. (a) and (b) is result from 98C pin1, (c) and (d) is result from 126C pin1, respectively.

concentration of substrate A. After second column, from third column, dye's concentration has been increasing dramatically with slightly reducing by the last wash. From this data, it is expected that at first two column contains mostly with unlabeled protein with labeled protein, while other columns demonstrate that free dyes has started to washed off together on filtered solution. Therefore, labeled protein from first 2 filtered samples are used for smFRET experiment.[5]

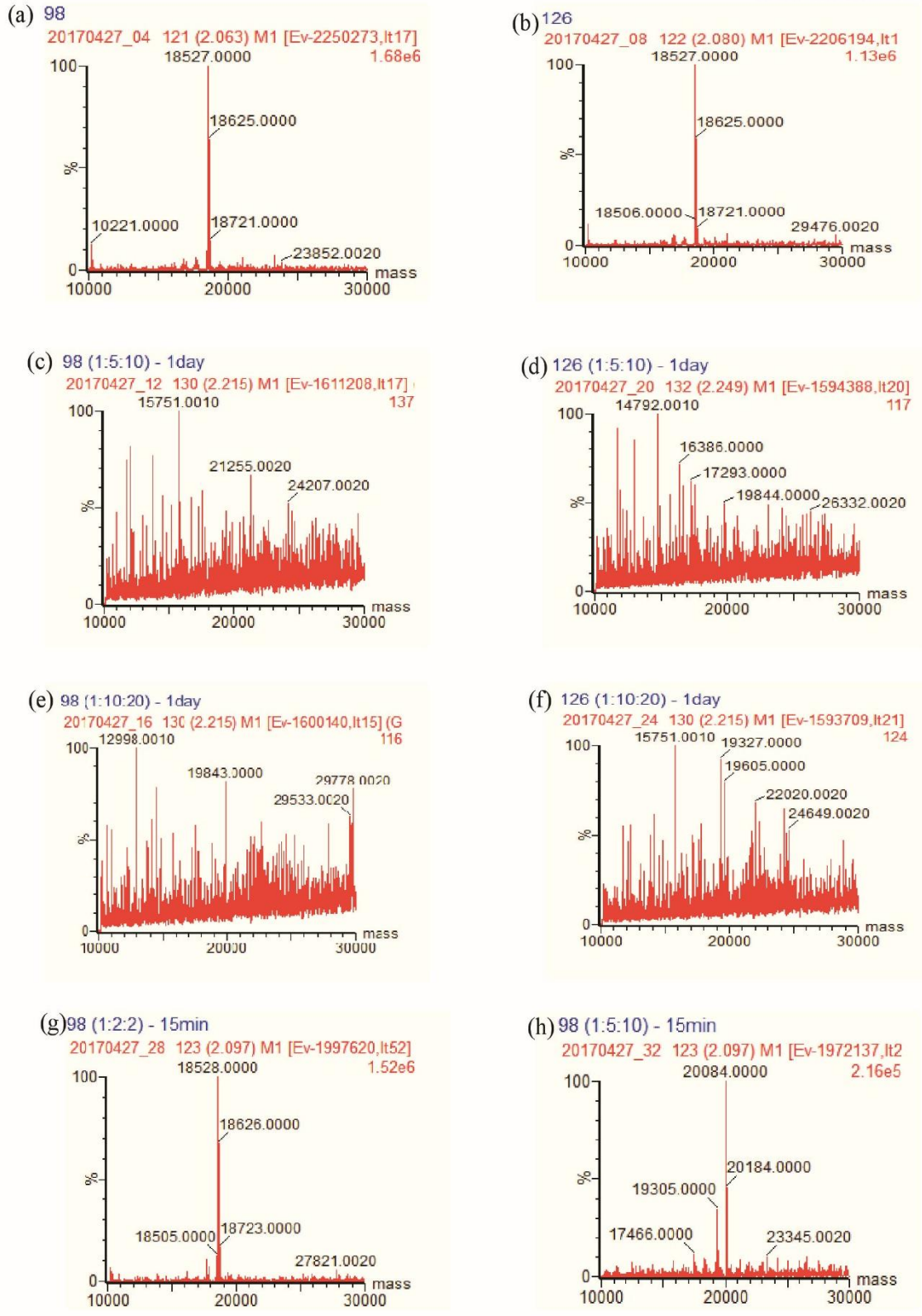
2.3. Qualification of materials

To verify if the first two solution containing labeled protein, mass spectrometry is used as very direct method. By measuring molecular weight, there can be no harder evidence to support it. Here is the principle how we get mass information. It composed of 4 part; (1) injection, (2) ionization, (3) deflection, and (4) detection. Once you inject the sample in aqueous solution, it is vaporized and ionized through electron beam. Electrically heated coil evaporates the sample, giving electrons, so that sample is bombarded with the stream of electrons. The electrons are captured on electron trap, only positive ions are going through the chamber where deflection occurs. In this chamber, these positive ions go under electromagnet, and this deflection is decided by two factor; mass and charge, by ratio of *m (mass) / z (charge)*. And the ions sorted by this magnetic field are detected and amplified, therefore giving mass information.



[Fig. 7] Mass Spectrometry
This figure shows how mass spectrometry composed of. Once sample is injected, through ionization and deflection, mass information is measured through detection. The deflection occurs according to m/z, when m stands for mass and z stands for charge. The mass is analyzed by detection tool, amplifying the signal and finally come to user the data.

To know which condition of environment for labeling gives the best efficiency, I have conducted labeling on different factors – for time, labeling ratio, temperature for labeling **(fig.9)**. Protein's mass is measured to be around 18527 Da, and cy3 and cy5 mass is 765.95 Da and 791.95 Da, respectively, as mentioned in previous section. The labeling was conducted as following conditions; (a) native 98C, (b) native 126C, (c) n(98C) : n(cy3) : n(cy5) = 1: 5 : 10, reaction on RT for an hour and labeling overnight under 4℃, (d) same condition as previous information but for 126C, (e) n(98C) : n(cy3) : n(cy5) = 1: 10 : 20, reaction on RT for an hour and labeling overnight under4℃ , (f) same condition as previous information but for 126C, (g) n(98C) : n(cy3) : n(cy5) = 1 : 2 : 2, reaction on RT for 15 mins and purification, (h) n(98C) : n(cy3) : n(cy5) = 1 : 5 : 10, reaction on RT for 15 mins and purification. In all these conditions, from condition (h), we could get very precise mass of expected measurement which is 20084.9 = 18527 (pin1) + 765.95 (cy3) + 791.95 (cy5). [6]

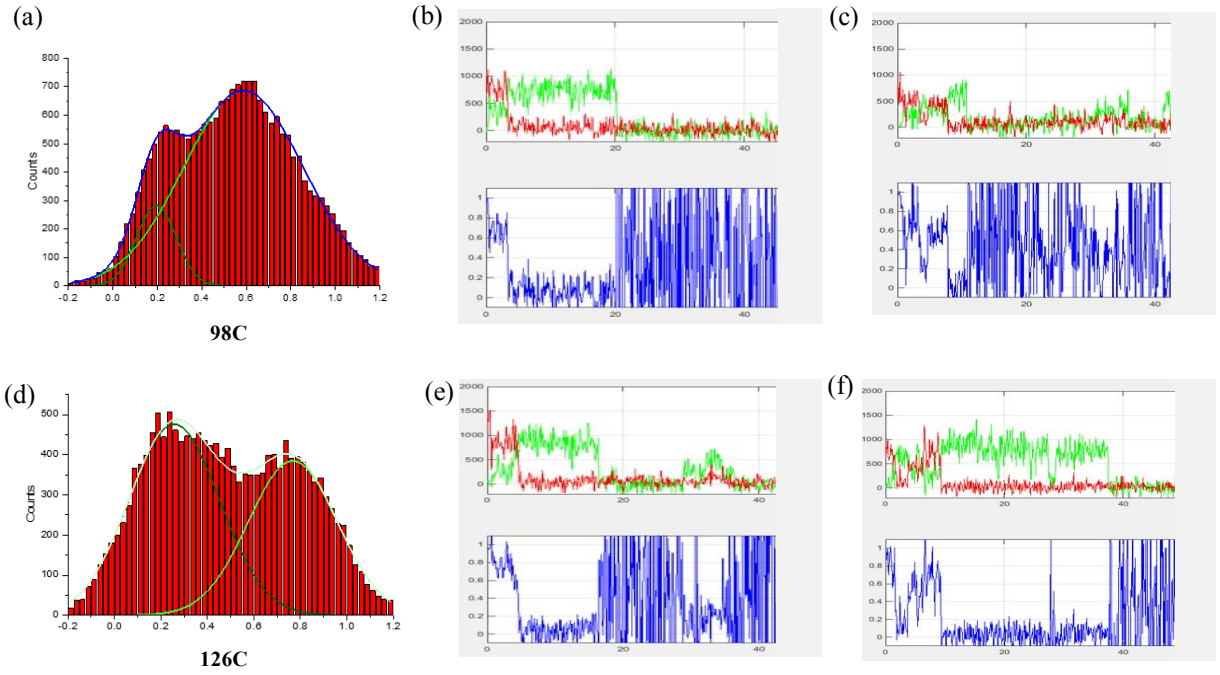


[Fig. 8] mass spectrometry result

(a), (b) shows mass information of native protein itself, while other figures from (c) to (h) shows mass information under different conditions. On overnight incubation, it shows huge noise for analyzing while without overnight incubation shows more stably labeled proteins with dyes. Also, the ratio between protein and dyes matters a lot if you compare (c) and (h), hence we can say condition (h) fits the best for labeling pin1 with maleimide dyes.

III. Result and Discussion

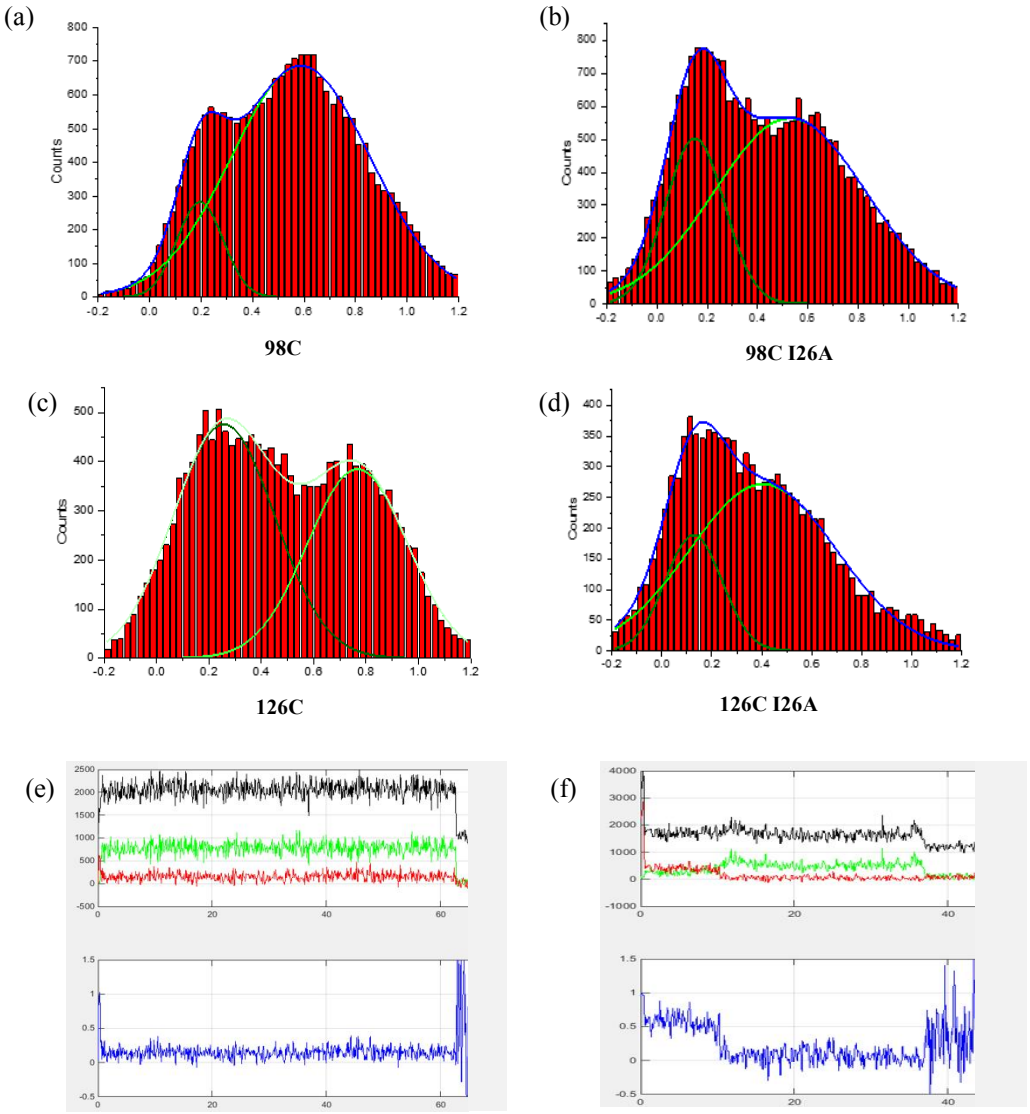
3.1 Inter-Domain Dynamics of native protein



[Fig. 9] FRET efficiency with example traces for native protein, pin1 for 98C and 126C
(a) is FRET efficiency of 98C, with example traces mostly observed (b) and (c). It is observed stably high FRET signal mostly with active dynamics back on forth around 0.2 FRET and 0.8 FRET. (d) is the one for 126C, with (e) and (f) as its example traces.

According to FRET result, the pin1's domain interaction is expected to be dynamics are actively observed, especially on 98C with higher FRET efficiency. This result demonstrates that interactive inter-domain dynamics occurs between two domains, WW domain and catalytic domain, which is within measurable unit for FRET experiment. This result will be standard to compare and to understand inter-domain dynamics according to change on environment of protein; I26A mutant, urea condition, peptide condition.

3.2. linker contributes freeier inter-domain dynamics

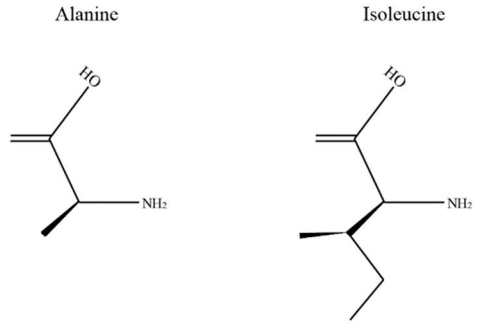


[Fig. 10] I26A mutant on pin1

(a) and (c) is attached to make understand easier by direct comparison of native protein dynamics and I26A mutant condition on pin1. (b) is I26A mutant on 98C and (d) is the one for 126C. (e) and (f) is extracted example trace which is frequently observed from I26A mutant proteins.

I26A is known to be conservative mutant, so that it can be used to understand some dynamics on proteins. There are few families of proteins which has similar or very same characteristics, Isoleucine and Alanine can be one of the case that is applied for this experiment. Isoleucine and Alanine has only one carbon chain difference, Alanin with one less carbon tail, but no huge difference on their characteristics; both aliphatic and branched hydrophobes (fig.12). So by

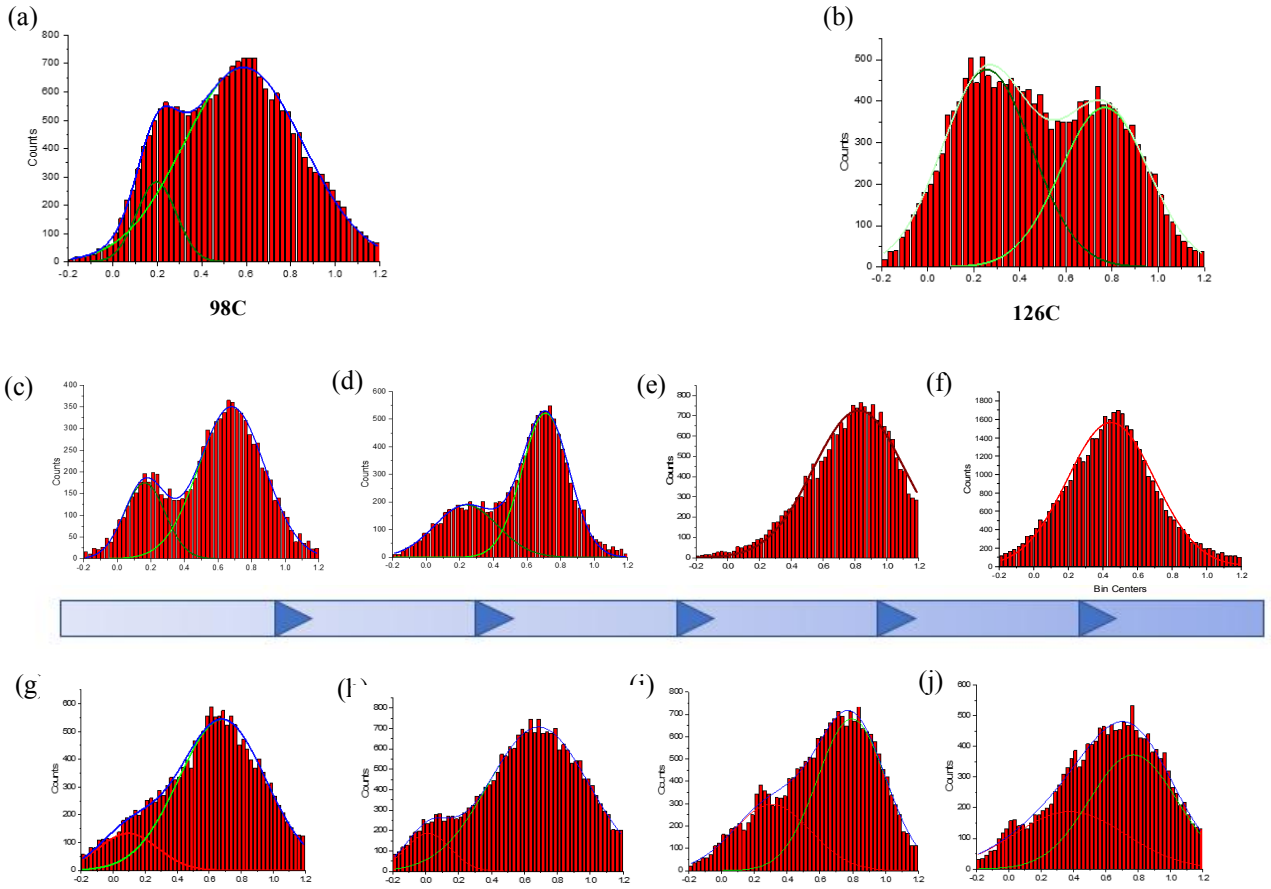
mutating Isoleucin 26 to Alanine (I26A), the design is expected to observe if the linker, since Isoleucine 26 is located on linker position.



[Fig. 11] Alanine vs. Isoleucine
Conservative mutation occurs naturally, when non-conservative mutation is mostly removed by natural selection. Alanine and Isoleucine are grouped to be in same category which are aliphatic with hydrophobic side chain – but only difference is coming from carbon chain they have

When you look at the FRET, overall from both 98C and 126C with I26A mutant's FRET ratio on low FRET and high FRET' ratio has been more likely condensed on low FRET tendency. This explains that linker's role on this dynamics are non neglectable when considering this inter-domain dynamics that with shorter linker, the dynamics are limited therefore showing low FRET more likely but still their main peak doesn't move a lot. [7]

3.3. Urea denature less compact region, showing naked dynamics



[Fig. 12] urea condition for pin1

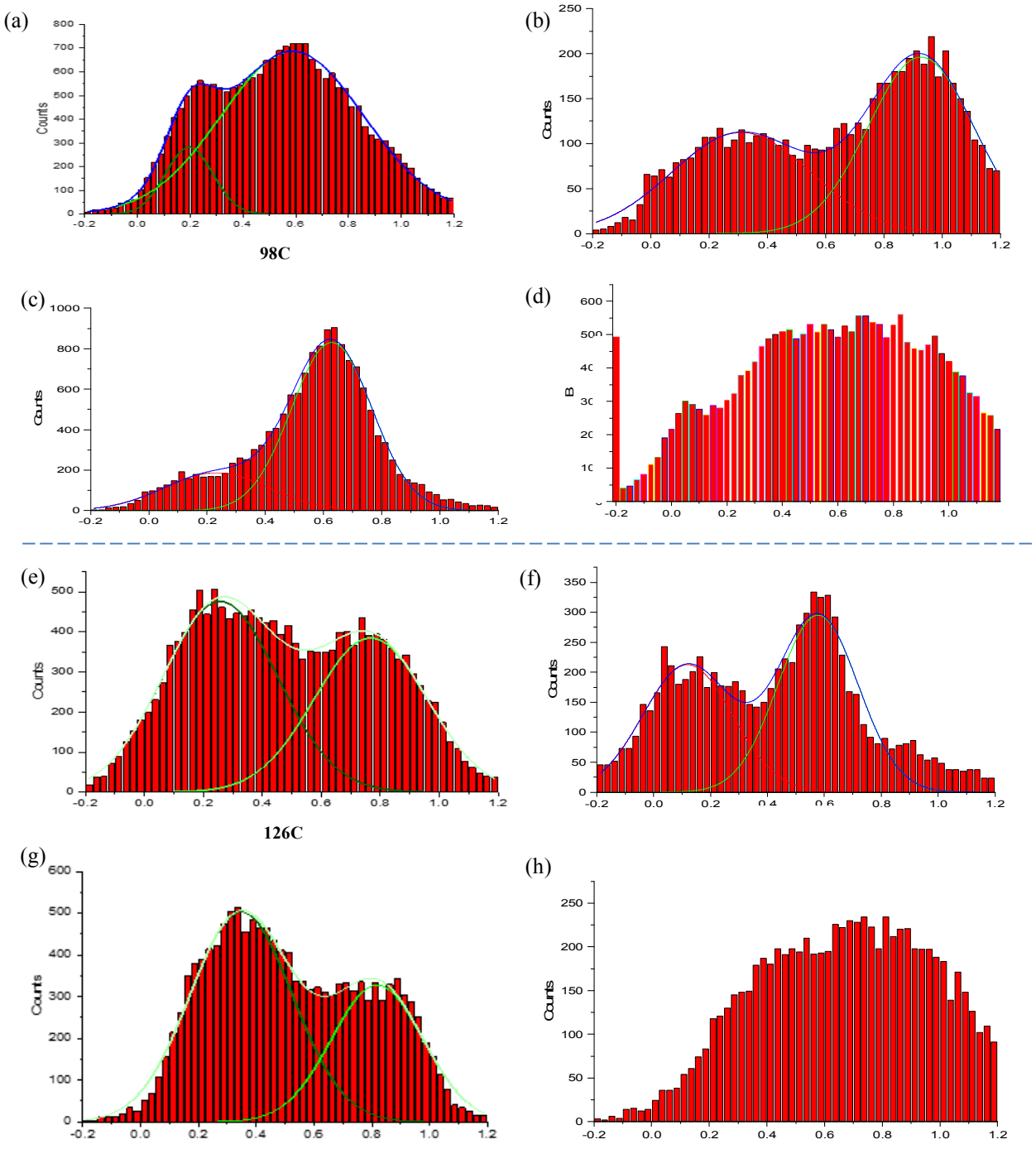
Each (a) and (b) is 98C and 126C FRET for direct understanding how FRET tendency are changing along with gradually higher concentration of urea on pin1. From (c) to (f) is 98C pin1 with urea condition, gradually from 0.1M to 0.5M, where (g) to (j) is the one for 126C pin1.

Urea has been studied to denature certain conformation on proteins in many studies. It may be very dangerous tool to be used when it is over-injected, but in other words, it can be used as a method to understand and to observe which linkage or interaction of certain mechanism is weaken, therefore the native or favorable dynamics of molecules. In many macro-molecule cases, the concentration of urea has been used up to 8M, but here we used relatively very small concentration of urea is used, still showing very significant changes on FRET tendency.

The higher concentration of urea, 0.1M to 0.3M of urea, the higher FRET efficiency has been detected, while from 0.5M urea concentration, it seems FRET efficiency has been ambiguously merged (showing around 0.5 FRET efficiency) and get lowered with higher concentration of urea.

This can be an evidence of that weaker FRET, further distanced interaction – mostly from not stable high FRET tendency but from actively changing dynamics observed, is less favorable, when higher and stable FRET tendency has strong position in this pin1. [8]

3.4. pintide, barrier of the inter-domain dynamics



[Fig. 13] pintide inserted pin1 dynamics in vesicle.

(a) and (e) is 98C and 126C pin1 interdomain dynamics, while (b) and (f) are the same dynamics observed, but not on surface attached but from vesicle experiment. (c) and (d) is the data when pintide is added 10uM and 100uM respectively while (g) and (h) is that for 126C. Pintide's Kd value is known to be 50uM.

Pintide, a substrate known to be interact with pin1, has an inhibitor-like activity on our protein of interest. Pintide is a ligand mimic which competes for binding of a native ligand to the WW-domain of Pin1. Competitive inhibition is characterized by the ability of the phosphorylated ligand mimic to compete, alter or prevent the WW-domain containing polypeptide from interacting with its native ligand. Likewise binding interactions between the WW-domain of Pin1 and phosphorylated ligands can be enhanced by phosphorylation of specific amino acid residues in the WW-domain and target ligand.

Its dissociation value, Kd value is known to be 50uM, which indicates that at 50uM concentration of pintide on pin1 environment, half of the substrate is interacting with Pin1, by the theoretical understanding. Therefore, we designed to make a concentration of pin1 much less and much more than Kd value, each 10uM and 100uM. Also, to see native dynamics of Pin1 with pintide substrate, we conducted this experiment on vesicle design, provided with pin1 inter-domain FRET efficiency on surface design as well.

With less concentration of pintide, 10uM environment, as it is shown very dramatically that there are huge loss on high FRET efficiency area, whereas low FRET ratio has been extremely increased. This can be interpreted as strong evidence of pintide, indeed as a competitive inhibitor for inter-domain interaction of Pin1. On the other hand, under 100uM of pintide circumstance, it is shown to have very broad and very non-specific, ambiguous dynamics occurred and interpreted on its result. This asks for further research collaborate with NMR or SAXS, which gives distance information on target spots on protein of interest, to support higher and harder evidence to figure out its dynamics. [9]

IV. Conclusion

Pin1, peptidyl-prolyl trans-cis isomerize by post phosphorylation, is studied in this research, but most of proteins with mobility are known to have one or more intrinsically disordered regions. Those disordered region's distortion can crucially effect on function of proteins, and finally are able to led to critical diseases. In case of the protein Pin1, their abnormal expression are led to cancer, Alzheimer's disease, or mal-immunity related disease, which are very critical diseases. Until now with ordinary studies, such as NMR or SAXS, couldn't directly figure out the dynamics and have certain limits, but this study shows direct understandings on inter-domain dynamics of Pin1. This can led into fundamental guidelines for intrinsically disordered proteins' dynamics and can be potentially applied for providing new pathway of cure the diseases.

In the view of academically, this research provides single molecule studies on this field which was done only with secondary, which means not so direct researches for studying protein dynamics, and can eventually led to understand dynamics for general and a number of proteins which are still under their veils.

Eventually, it can also provide the new approach for drug screening, which are caused by pin1 protein or pin1-similar proteins. Unlike current screening, high cost (cost-inefficient) investment on the experiment without knowing actual molecular dynamics, this is very direct on single-molecule size of understanding and can create some opportunities which can be cost-efficient for developing drug targeting with higher accuracy.

V. References

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ACKNOWLEDGEMENT

I am truly grateful for all opportunities I could achieve during last years which allowed me to accomplish one big step on my academic career here. I had an extraordinary supervisor professor, Hajin Kim, who guided not only with my research guidance, but also with warm hearted manners towards students. Also thanks to committee members, Jung-Min Kee and Jayil Lee.

I had amazing supporters who encouraged me not to give up and to achieve it. Special thanks to my Central Asian friends, Korean friends along with UNISCA members with Jennifer and Sunny, indeed extraordinary experience as a student from biomedical engineering field. All friends who cheered me up surely deserve all my gratitude.

마지막으로 사랑과 격려로 지켜봐 주신 부모님과 가족들에게 다시 한 번 감사함을 표현하고 싶습니다.